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(71) Applicant *(for all designated States except US)*: DALGETY PLC [GB/GB]; 100 George Street, London W1H 5RH (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: ANDERSSON, Leif [SE/SE]; Bergagatan 30, S-752 39 Uppsala (SE). MOLLER, Maria, Johansson [SE/SE]; St Johannesgatan 31A, S-753 22 Uppsala (SE). WALES, Richard [GB/GB]; Dalgety plc, Food Technology Centre, Station Road, Cambridge CB1 2JN (GB). SIGGENS, Kenneth, William [GB/GB]; Dalgety plc, Food Technology Centre, Station Road, Cambridge CB1 2JN (GB). PLASTOW, Graham, Stuart [GB/GB]; PIC Group, Fyfield Wick, Abingdon, Oxfordshire OX13 5NA (GB).

(74) Agents: CHAPMAN, Paul, William et al.; Kilburn & Strode, 30 John Street, London WC1N 2DD (GB).

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(54) Title: METHODS FOR DETERMINING THE COAT COLOUR GENOTYPE OF A PIG

(57) Abstract

Methods for determining the coat colour genotype of pigs are provided. In addition, kits comprising suitable reagents for use in the method are provided and specific primers for use in PCR based methods are disclosed.

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METHODS FOR DETERMINING THE COAT COLOUR GENOTYPE OF A PIG

The present invention relates to methods of screening pig nucleic acid to determine pig genotype with respect to coat colour, and to kits for use in carrying out such methods.

White is the predominant coat colour among European commercial pig breeds e.g. Large White and Landrace. However, there are a number of commercially important coloured breeds, demonstrating a number of colours and combinations. The Duroc, associated with meat tenderness, is red, the Pietrain, which is a heavily muscled animal which produces a very lean carcass, is spotted, and the prolific Chinese Meishan breed is black.

Coat colour is important to the pig breeding industry for a number of reasons. Firstly, in a number of markets there is a preference for white skinned meat. This is due to the fact that pork is often marketed with the skin still attached, and skins from coloured pigs, even if dehaired, can still exhibit coloured hair roots, which can lead to a negative perception by the consumer, since the surface of the meat may appear to be spotted by mould. It is necessary, therefore, in these markets, to remove the skin from such carcasses, entailing additional cost. For example, in the US, coloured carcasses are associated with approximately 1% of skin defects requiring dehairing and skinning to remove pigment. As a result of this, coloured pig carcasses are generally discounted.

Secondly, gross variation in the appearance (i.e. a range of coat colours) of pigs claimed to be genetically

consistent for traits other than coat colour can lead to questions about the consistency and quality of the animals in the mind of pig-producing customers.

In addition, pig breeders would like to be able to be in a position to ensure consistency in breeding populations. Thus, breeders may wish to ensure that progeny produced by breeding crosses were always white. Alternatively, a breeder of Pietrain pigs may wish to ensure that breeding crosses always produced the characteristic Pietrain colouring. Traditional animal breeding practices have, in the past, been used to attempt to eliminate colour (other than white) from pig lines.

The gene determining whether the animal is coloured or the desired white is designated I (for inhibition of coat colour). The version of the gene preventing the expression of any colour (I) is dominant to that which allows colour to develop (i). Traditional selection for white animals has reduced the frequency of i, but it still remains in the population in white heterozygous carrier animals. These animals can only be identified when they produce coloured offspring through matings with other heterozygous animals. Only through a programme of test matings can heterozygotes be identified which would enable the recessive allele to be eliminated from a given population. Such a programme would be time-consuming and costly and as such is not cost effective. Thus, i/i animals will inevitably be produced.

In addition, the situation is complicated further by the existence of another allele of I called I^P (I-patch). The I^P allele is recessive to I but is dominant to i. Thus, animals which have the genotype I^P/I^P or I^P/i will show

patches of colour.

Using a reference family developed from crosses between the European wild pig and a large white breed (Swedish Yorkshire), the position of the *I* gene on the porcine genetic map has been determined. The gene is located on chromosome 8 in the pig, close to the genes for albumin and for the α-subunit of platelet-derived growth factor (PDGFRA) (Johansson *et al*, *Genomics* **14**: 965-969 (1992)). The mouse genetic map includes a homologous region located on mouse chromosome 5. This region contains a number of genes playing a role in the determination of mouse coat colour, namely *W* (dominant white spotting), *Ph* (patch) and *Rw* (rump white). The mouse *W* gene has been shown to co-locate with the *KIT* gene and some mutant genotypes at the *W* locus are due to structural changes in the *KIT* gene (Chabot *et al*, *Nature* **335**: 88-89 (1988), Geissler *et al*, *Cell* **55**: 185-192 (1988) and Nocka *et al*, *EMBO Journal* **9**: 1805-1813 (1990)).

We have now found that the *KIT* gene in pigs is involved with coat colour determination. More particularly, we have found that the difference between *I*, or I^P, and *i* is duplication of at least part of the *KIT* gene in the *I* or I^P allele. This duplication can result in two or more copies of a particular region of the *KIT* gene being present.

Thus, this has allowed us to develop methods of distinguishing between the alleles *I*, I^P and *i*, and thus for determining the genotype of individual pigs with respect to coat colour.

Therefore, in a first aspect, the present invention

provides a method of determining the coat colour genotype of a pig which comprises:-

(i) obtaining a sample of pig nucleic acid; and

(ii) analysing the nucleic acid obtained in (i) to determine whether duplication of all or part of the *KIT* gene is present.

The presence of duplication in the *KIT* gene sequence indicates the presence of either the *I* or I^P allele. In some pig populations it is known that the incidence of I^P is low or indeed non-existent. In such populations determining the presence or absence of duplication will be sufficient to provide a reasonable degree of confidence concerning a particular pig's genotype. Thus, by means of simply determining the presence or absence of duplication of the *KIT* gene (either complete or part thereof), coat colour genotype of a particular pig can be determined with a reasonably high degree of certainty.

However, in other populations it will be necessary to distinguish between the presence of *I* and I^P.

We have found that although both *I* and I^P have a duplication in the *KIT* gene, only *I* and not I^P exhibits a deletion in one of the duplicated regions. It is therefore possible to distinguish between these alleles on that basis.

Thus, the method may further comprise the step:

(iii) determining whether the duplication is due to the presence of *I* or I^P.

Suitably, this determination is made by analysing for the presence or absence of a deletion in at least one of the duplicated regions.

Suitably, the method of the invention will be carried out on pig genomic DNA, although pig RNA may also be analysed to determine the presence or absence of duplication in the *KIT* gene.

There may be a number of effects on the production of RNA from this gene, resulting from the duplication of part of the DNA sequence. These could include the inhibition of the production of RNA, alteration of the level of synthesis of the RNA, alteration in the size or processing kinetics of the RNA or alteration in the distribution of RNA production throughout the body of the animal. There might also be effects on the production of RNA from other genes caused by epistatic effects of the duplication.

Preferably, the determination carried out in step (ii) involves the use of PCR techniques, using an appropriate pair of primers. PCR, or polymerase chain reaction, is a widely used procedure in which a defined region of a DNA molecule can be amplified *in vitro* using a thermostable version of the enzyme DNA polymerase. Two known sequences that flank the region to be amplified are selected and priming oligonucleotides synthesised to correspond to these regions. If the primers are located sufficiently close together on the same piece of DNA, the region between them will be amplified. A polymerase chain reaction consists of a number of cycles of amplification. Each cycle begins with a denaturation step, typically at 94°C, in which the two strands of the

template DNA molecule are separated. The temperature is then dropped to a temperature at which the synthetic oligonucleotide primers can anneal to the template (typically 50-60°C). Through the high concentration of primers relative to template, the primers anneal to the template before template-template hybrids form. The annealing temperature is chosen such that annealing only occurs to the complementary regions of DNA within the template, and not to other regions of imperfect complementarity. The temperature is then raised to 72°C, at which the thermostable DNA polymerase can extend the bound primer, thus producing the strand of DNA complementary to the template.

In the earlier stages of the reaction, each cycle results in two-fold amplification of the template present. As each of these newly synthesised strands can function as template, the increase in molecules corresponding to the defined region is exponential. However, in the later stages of the reaction (typically 25 cycles onwards) the amplification ceases to be exponential due to depletion of reaction constituents (e.g. primers), and the increased concentration of template molecules leading to increased template-template hybrid formation at the annealing stage. In such reactions, the amount of product is directly related to the amount of template initially present, but only in the exponential phase. It is critical in quantitative applications as described herein that the number of cycles used ensures that the reaction remains within the exponential phase.

The PCR can be used in several ways to determine whether or not duplication of the *KIT* gene is present. Firstly, the primers can be chosen such that amplification of a

portion of the *KIT* gene duplicated in *I* or I^P and not in *i* are used. This PCR is compared to a second PCR using primers which allow amplification of a control sequence which is known to be present in only a single copy in any chromosome. Comparison of the ratios of the individual PCR reaction products will enable an estimation of the duplication of the *KIT* region present, if any. Clearly, if it is assumed that the region of DNA in question is present in two copies in the *I* or I^P and in only one copy in the *i* allele, then the ratios of *KIT* product to control product expected will be as follows:-

GENOTYPE	*KIT*/CONTROL
I/I	2
I^P/I	2
I^P/I^P	2
I/i	1.5
I^P/i	1.5
i/i	1

In practice, the ratios obtained may vary from this due to differences in the reaction kinetics of the two amplification reactions which are occurring.

Pairs of suitable primers for use with the above described method include:-

GAATATTGTTGCTATGGTGATCTCC *KIT*1-FOR
CCGCTTCTGCGTGATCTTCCTG *KIT*1-REV
and
GG(C/T)AATCACATGAATATTGTGAA *KIT*2-FOR
TCACCATAGCAACAATATTCTGT *KIT*2-REV
and
TC(A/G)TACATAGAAAGAGA(C/T)GTGACTC *KIT*3-FOR

CCTTT(A/G)ACCAC(A/G)TAATT(A/C)GAATC *KIT*3-REV
and
GTGATG(A/G)T(G/T)CT(C/G)ACCTACAAATA *KIT*4-FOR
GTCTATGTAAACATAATTGTTTCC *KIT*4-REV

As described above, the inclusion of a control PCR reaction allowing direct comparison to determine the ratio of amplified product is preferred.

This is suitably achieved by reference to a control sequence chosen because it is known that the pig chromosome carries only a single copy. Thus, by employing suitable primers for that control sequence, a PCR product can be generated and quantified. Comparison with the *KIT* gene PCR product thus provides a direct reading of the degree of duplication.

One example of a suitable control sequence is part of an exon of the muscle calcium release channel gene (CRC) and a suitable pair of primers is:-

CTGGATGTCCTGTGTTCCCTGT CRC FORWARD
AGGTTTGTCTGCAGCAGAAGCTC CRC REVERSE

Another example of a suitable control sequence is part of the porcine interferon-β gene (Artursson *et al*, *Journal of Interferon Research* **12**: 153-160 (1992)), and a suitable set of primers is:

GATGAACTTTGAGGTCCCTGAGGAG IFN-β Forward
TTTCTTCTGAGAATGCCGAAGATCTG IFN-β Reverse

Other suitable sequences for control primers include regions from the gene for the common α subunit of porcine

pituitary glycoprotein hormone (Kato *et al*, *Journal of Molecular Endocrinology* 7: 27-34 (1991)), the gene for the β subunit of porcine luteinizing hormone (Ezashi *et al*, *Journal of Molecular Endocrinology* 5: 137-146 (1990)) or any other single copy porcine gene.

Most preferably, the PCR for the *KIT* gene and that for the control sequence are carried out simultaneously on a single sample of pig DNA.

A second method for determining whether any duplication of the *KIT* gene (including a part thereof) is present relies on the fact that at the boundary of any duplicated region there will be present a nucleotide sequence unique to the *I* allele. Therefore, by utilising primers specific for such boundary nucleotide sequences, or junctions, it is possible to determine the frequency of the *I* allele.

A third method for the determination of the structure of the *KIT* gene is to use a linked genetic polymorphism which is closely associated with the presence or absence of the duplication. Such a polymorphism may occur in the *KIT* gene itself or in a chromosomal region linked to *KIT*. By using a single linked marker in complete association with the presence/absence of the duplication or a combination of markers showing a partial association a highly informative test can be developed. For instance, the SSCP (Single Strand Conformation Polymorphism) method may be used to develop such polymorphism. The principle of the method is that double-stranded DNA, produced by PCR, is denatured into single-stranded DNA which is then separated by non-denaturating gel electrophoresis. Under non-denaturating conditions the single-stranded DNA forms

a secondary structure due to intrastrand interaction but a proportion of the single-stranded DNA will renature and form double-stranded DNA. Two types of polymorphism may be revealed by this method. Firstly, a difference in nucleotide sequence between two alleles may influence the secondary structure of single-stranded DNA which is revealed as a difference in the mobility rate during electrophoresis. Secondly, a difference in nucleotide sequence often influences the mobility of the heteroduplex DNA (A heteroduplex is a double-stranded DNA molecule formed by two single-stranded molecules representing different alleles).

A fourth method of determining the structure of the *KIT* gene in relation to the number of copies of the region subject to duplication involves the use of pulsed field gel electrophoresis. Pulsed field gel electrophoresis being a technique in which the size of large DNA fragments can be analysed. In this application the process would be to utilize a restriction endonuclease that cleaved the genomic DNA at specific sites flanking the region found to be duplicated in the DNA of animals carrying the *I* allele of the *KIT* gene. Genomic DNA cleaved with such an enzyme would be subject to pulsed field electrophoresis followed by transfer to a DNA binding membrane. A probe specific for the region subject to duplication could then be used to determine the original location on the gel, and therefore the size of that fragment by comparison to suitable DNA size standards. Should the DNA from an animal contain a duplication of a portion of the *KIT* gene, this specific fragment would be increased in size. Heterozygous animals will be found to show two differently sized specific bands, the smaller representing the non duplicated allele

i, the larger representing the duplicated allele *I* or I^P. This technique will also show alleles containing more than two copies of the duplicated region through the presence of fragments having a further increase in size by the unit length of the duplication.

In a second aspect, the present invention provides a method for determining the coat colour genotype in pigs, which comprises:-

(i) obtaining a sample of pig genomic DNA;

(ii) hybridising the genomic DNA from (i) with one or more suitable primers;

(iii) carrying out one or more PCR cycles using the hybridised nucleic acid from (ii); and

(iv) determining the amount of PCR reaction product.

The method of this aspect may also include a further step:

(v) determining whether any duplication present is due to the presence of *I* or I^P.

Suitably, this determination is carried out by analysing for the presence or absence of a deletion in at least one of the duplicated regions.

Association between genetic markers and genes responsible for a particular trait can be disrupted by genetic recombination. Thus, the closer the physical distance

between the marker and the gene in question, the less likely it is that recombination will separate them. It is also possible to establish linkage between specific alleles of alternative DNA markers and alleles of DNA markers known to be associated with a particular gene (e.g. the *KIT* gene discussed herein), which have previously been shown to be associated with a particular trait. Thus, in the present situation, taking the *KIT* gene, it would be possible, at least in the short term, to select for pigs with a particular coat colour, indirectly, by selecting for certain alleles of a *KIT* gene associated marker through the selection of specific alleles of alternative chromosome 8 markers. Examples of such markers known to be linked to the *KIT* gene on porcine chromosome 8 include genetic polymorphism in the *KIT* gene itself or in the closely linked genes for the α-subunit of platelet derived growth factor (PDGFRA) and albumin.

Particular genetic markers associated with the *KIT* gene are microsatellites. These are simple sequence repeats of 4, 3 or, more usually, 2 nucleotides, which occur essentially at random around the genome at approximately every 50,000 bases (about 60,000 microsatellites per haploid genome). Stuttering of DNA polymerase during replication and unequal crossing-over during recombination are thought to result in the loss or gain of repeat units. This means that microsatellites are usually polymorphic and can have several repeat length alleles.

Examples of linked microsatellite sequences include S0086 (Ellegren *et al*, *Genomics*, **16**:431-439 (1993)) , S0017 (Coppieters *et al*, *Animal Genetics* **24**: 163-170 (1993)),

Sw527, Swr750 and SW916 (Rhorer *et al*, *Genetics*, **136**:231-245 (1994)) . It would be possible to select indirectly for alleles of the *KIT* gene linked to coat colour using any of the above markers, or indeed any other linked markers on porcine chromosome 8.

As discussed herein, the present invention relies upon a determination of *KIT* gene DNA sequence copy number. To that end a nucleotide probe representing the duplicated *KIT* segment, or part of it or indeed any other nucleotide probe showing sufficient similarity to such a porcine probe may be used. For example, the following methods can be used to carry out such a determination:

(i) using nucleotide probes derived from nucleotide sequences of at least part of the DNA of the *KIT* gene, and RNA derived from it, from, e.g. mouse (Gokkel *et al*, *Oncogene* 7, 1423-1429 (1992)) and/or man (Giebel *et al*, *Oncogene* 7, 2207-2217 (1992)). Such probes, due to conservation, would hybridise to the pig gene;

(ii) where the amino acid sequence of the *KIT* protein of an animal is known, the possible nucleotide sequences of the DNA encoding that protein, or portions of it, can be deduced. Based on that, mixed oligonucleotide preparations can be used as probes for the pig *KIT* gene;

(iii) probes can be designed based on the protein sequences (and corresponding nucleotide sequences) for proteins that have functional

homc_ogies to the whole or part of the *KIT* gene, for example *v-KIT* (Besmer *et al*, *Nature* 320: 415-421 (1986)).

All of the probes derived as described above may be used to probe animal derived nucleic acid preparations transferred to suitable matrices for hybridisation such as Nylon membranes (e.g. Hybond N Amersham Internationa_) by Southern, northern or dot blotting. The ratio of the amounts of the *KIT* and control probes hybridising to the matrix bound nucleic acid can be used to determine *KIT* copy numbers. The amount of bound probes can be quantified through labelling the probes with radioactive isotopes. Other, non-isotopic nucleic acid labelling kits are now available and can also be used.

The reverse of the procedure involving hybridisation of animal derived nucleic acid to matrix is also possible. In this, probe is bound to the matrix and used to capture, through a hybridisation protocol, genomic DNA or RNA labelled in such a way as previously described, thus allowing quantitation of the amount bound. The amount bound is, if the conditions are correct, related to the total amounts (or copy number) of the *KIT* and control nucleic acid sequences present.

Other methods of quantifying PCR amplified DNA include radiolabelling based methods. An example is radiolabelling of one or both of the oligonucleotide primers, followed by quantitation of the radioactivity in the PCR product through densitometry of autoradiographs of DNA gels. An alternative procedure is the differential labelling of the oligonucleotides for the two products of the PCR reaction with different isotopes

allowing quantification of each separate product after removal of unincorporated labelled oligonucleotides through precipitation, filtration, differential centrifugation or other procedures. PCR product can also be quantitated using other staining procedures utilising dyes such as ethidium bromide or SYBR green (Molecular Probes, Inc.) in combination with densitometry or fluorimetry.

Yet another method of quantifying the products of a differential PCR in which two PCRs proceed in the same tube to produce two separate products, as described in this patent, is the use of the TaqMan™ system (Perkin Elmer Corp.). In this system, in addition to the two oligonucleotide primers flanking the region to be amplified a third oligonucleotide probe is used that binds to the amplified region. The flanking primers are unlabelled while the probe carries two fluorescent labels. On the 3′ end of the probe is a reporter dye, the fluoresence of which is quenched by a separate fluorophore attached to the 5′ end of the probe. During PCR this probe binds to the product DNA molecules. As PCR proceeds these products are used as templates during which the Taq DNA polymerase cleaves off the 5′ quenching dye of the probe as it displaces it. This removal of the quenching agent allows fluoresence from the reporter dye to be detected. The degree of fluoresence is proportional to, and therefore a measure of, the amount of PCR product produced. A reaction may include two separate sets of PCR primers and two probes, each corresponding to a separate genomic DNA region. In this way, as long as the criteria for quantitative PCR are obeyed, the relative amounts of each template region can be measured.

Protein nucleic acids might be used in any of the above hybridisation procedures (PerSeptive Biosystems, Inc., Cambridge, MA).

In a third aspect, the invention provides a method for determining the coat colour genotype of a pig, which method comprises:

(i) obtaining a sample of pig *KIT* protein; and

(ii) analysing the protein obtained in (i) to determine whether duplication of all or part of the *KIT* gene is present.

Antibodies raised against epitopes of *KIT* or related proteins could be used to determine the level or form of the c-*KIT* proteins present in the animal through Western blotting or ELISA procedures. Also antibodies could be raised against the different DNA structures comprising the various alleles of the *KIT* gene and the genotype determined through ELISA techniques.

In a fourth aspect, the invention provides a kit for determining the coat colour genotype of a pig, comprising one or more reagents capable of indicating the presence of duplication of all or part of the *KIT* gene sequence in pig genomic DNA.

It will also be appreciated from the discussion above concerning the difference between *I* and I^P, that this difference can also be exploited to distinguish between genotypes. As the deletion only appears to be present in one of the duplicated regions of *I* (where *I* contains two copies of the duplicated region) and not in *i*, it

provides a marker of *I*. Oligonucleotide primers can be designed to hybridize to genomic DNA either side of the position at which the deletion is found and PCR carried out, resulting in two possible sizes of product.

That obtained where the four base pair deletion is present will be four base pairs shorter than that from the same region where the deletion is not present. The relative amount of these two products can be determined by any of the methods previously discussed, but one preferred method involves having one of the oligonuleotide primers labelled with a fluorescent dye combined with electrophoresis in an apparatus with suitable detection equipment or the TaqMan apparatus (Perkin-Elmer).

One particular advantage of this testing strategy regarding the PCR component is that it is not so susceptible to loss of discrimination as the PCR leaves the exponential phase. The primers, their binding sites and the products are the same or similar for the non-deleted and deleted template regions, thus, the rate of production of one product relative to the other is unlikely to vary at different stages of the reaction. As the formation of template/template hybrids begins to compete with the formation of template/primer complexes it affects both sub-reactions simultaneously. The overall result is a high degree of similarity between the kinetics of the production of each product.

In a line of pigs containing only *I* or *i* and not I^P the ratio of the two products obtained can be related to the genotype as shown below, based upon the fact that each copy of the *I* allele gives rise to equal amounts of the

undeleted and deleted product while *i* gives only the undeleted product.

Genotype	Copies of undeleted region present	Copies of deleted region present	Ratio of undeleted to deleted product
II	2	2	1:1
Ii	2	1	2:1
ii	2	0	2:0
II^P	3	1	3:1
I^PI^P	4	0	4:0
I^Pi	3	0	3:0

In a fifth aspect, therefore, the present invention provides a method of determining the coat colour genotype of a pig which comprises:-

(i) obtaining a sample of pig nucleic acid; and

(ii) analysing the nucleic acid obtained in (i) to identify the presence or absence of a deletion in the *KIT* gene sequence.

Simply by identifying the presence or absence of the deletion, one can distinguish between *I* and *i*. Of course the presence of I^P complicates the results since, due to the absence of the deletion in one copy of the duplication I^P will effectively mimic *i* in such a test. Thus, the use of this method on lines carrying I^P may result in the misidentification of animals carrying this allele as *ii*. However, such a method may still find use, for instance in certain aspects of animal breeding where all that is required is the positive identification of

animals which are homozygous for *I*.

Of course, it will also be appreciated that each individual genotype either generates a different ratio of the two products, or produces different amounts of a single product type. Thus, such a test will also provide a means of determining the absolute genotype of an individual animal by means of quantification of the product produced. In such a case the amounts of DNA used in each test should be controlled in order to ensure that differences in the amount of product can be accurately determined.

Preferably, the determination step (ii) involves PCR amplification, usually on a sample of pig genomic DNA. Suitable pairs of primers which can be used in such a PCR method include:

TGTGGGAGCTCTTCTCTTTAGG *KIT*DEL1-FOR
CCAGCAGGACAATGGGAACATCT *KIT*DEL1-REV
and
GAAAGTGA(C/T)GTCTGGTCCTAT(C/G)GGAT *KIT*DEL2-FOR
AGCCTTCCTTGATCATCTTGTAG *KIT*DEL2-REV

One of numerous alternative ways of approaching this would be to use, in PCR, primers that bind to the unique nucleotide sequences created in each version of this region of the *KIT* gene through the presence or absence of the deletion. Such primers could be designed such that they only yielded a product in the presence of the corresponding sequence. Hence, if each of the two alternative primers was used with a different fluorescent label, separation by gel electrophoresis would not be required, identification of each product being made on

the basis of their fluorescence.

There is preliminary evidence based upon the inheritance of specific values for ratios obtained when testing for the presence of the duplication and the number of copies of the deletion that alleles of *I* containing more than two copies of the duplicated region exist in certain breeds of pig.

The discrimination between animals with these varying forms of *I* can be enhanced using a combination of the ratio arising from the determination of the number of copies of the duplicated region present with that from the determination of the presence of the deletion. The structure of the *KIT* gene in alleles that might be present given the existence of duplicated versions with more than two copies is shown below:

ALLELE	COPIES OF DUPLICATED REGION	COPIES OF DUPLICATED REGION CONTAINING DELETION
i	1	0
I^P (I 2.0)	2	0
I 2.1	2	1
I 3.0	3	0
I 3.1	3	1
I 3.2	3	2

The possible genotypes arising from the presence of these alleles and the respective ratios obtained in the determinations of the assay for the number of copies of the duplicated region and that for the number of copies containing the deletion are shown below:

GENOTYPE	RATIO: copies of *KIT* region/copies of control region	RATIO: copies of non deleted region/ copies of deleted region
i/i	1.0	0.0
I^P/i	1.5	0.0
I2.1/i	1.5	2.0
I3.0/i	2.0	0.0
I3.1/i	2.0	3.0
I3.2/i	2.0	1.0
I^P/I^P	2.0	0.0
I2.1/I^P	2.0	3.0
I3.0/I^P	2.5	0.0
I3.1/I^P	2.5	4.0
I3.2/I^P	2.5	1.5
I2.1/I2.1	2.0	1.0
I3.0/I2.1	2.5	4.0
I3.1/I2.1	2.5	1.5
I3.2/I2.1	2.5	0.67
I3.0/I3.0	3.0	0.0
I3.1/I3.0	3.0	5.0
I3.2/I3.0	3.0	2.0
I3.1/I3.1	3.0	2.0
I3.2/I3.1	3.0	1.0
I3.2/I3.2	3.0	0.5

One method by which the two tests may be combined involves the use of the TaqMan™ system (Perkin Elmer Corp.). In this specific application of TaqMan three probes are used with two sets of PCR primers. One probe allows the measurement of the control product arising from one of the sets of primers. the other set of PCR

primers allow the amplification of the region of the duplication that may or may not contain the deletion. The remaining probes detect either the deleted or non-deleted products from this amplification. From the data obtained two calculations can be made as bel;ow providing the values as obtained from the two separate tests described previously.

A: copies of *KIT* gene region:copies of control region

$$\frac{(\text{non-deleted } KIT \text{ gene product} + \text{deleted } KIT \text{ gene product})}{\text{control gene product}}$$

B: non deleted *KIT* gene: deleted *KIT* gene

$$\frac{\text{non-deleted } KIT \text{ gene}}{\text{deleted } KIT \text{ gene}}$$

Where appropriate, preferred features of each aspect of the invention are applicable to each other aspect *mutatis mutandis*.

The invention will now be described with reference to the following examples which should not be construed as in any way limiting the invention.

Example 3 refers to Figure 1 wherein:

FIGURE 1: shows the results of SSCP analysis.

EXAMPLE 1

(i) DNA Preparation

DNA can be prepared from any source of tissue containing cell nuclei, for example white blood cells, hair follicles, ear notches and muscle. The procedure outlined here relates to blood cell preparations; other tissues can be processed similarly by directly suspending material in K buffer and then proceeding from the same stage of the blood procedure. The method outlined here produces a cell lysate containing crude DNA which is suitable for PCR amplification. However, any method for preparing purified, or crude, DNA should be equally effective.

Blood was collected in 50 mM EDTA pH 8.0 to prevent coagulation. 50 μl of blood was dispersed into a small microcentrifuge tube (0.5 ml Eppendorf or equivalent). 450 μl of TE buffer was added to lyse the red blood cells (haem groups inhibit PCR) and the mix vortexed for 2 seconds. The intact white and residual red blood cells were then centrifuged for 12 seconds at 13,000 g in a microcentrifuge. The supernatant was removed by gentle aspiration using a low pressure vacuum pump system. A further 450 μl of TE buffer was then added to lyse the remaining red blood cells and the white blood cells collected by centrifugation as before. If any redness remained in the pellet, this process was repeated until the pellet was white. After removal of the last drop of supernatant from the pelleted white blood cells, 100 μl of K buffer containing proteinase K was added and the mixture incubated at 55°C for 2 hours. The mixture was then heated to 95-100°C for 8 minutes and the DNA lysates

stored at -20°C until needed.

Reagents.

TE buffer:	10 mM TRIS-HCl pH 8.0
	1 mM EDTA
K buffer:	50 mM KCl
	10 mM TRIS-HCl pH 8.3
	2.5 mM $MgCl_2$
	0.5% Tween 20

Prior to use for lysates, 10 µl of 20 mg/ml proteinase K (Molecular Probes Inc.) per 1.0 ml of K buffer was added.

(ii) <u>PCR</u>

Reactions were set up as follows in thin walled 0.25 ml tubes (Perkin Elmer):

4.0 µl 5 µM *CRC* Forward primer;
4.0 µl 5 µM *CRC* Reverse primer;
4.0 µl 5 µM *KIT1*-REV primer;
4.0 µl 5 µM *KIT1*-FOR primer;
4.0 µl 2 mM dNTPs (Pharmacia);
4.0 µl 35 mM $MgCl_2$.

A wax bead (PCR Gem 50, Perkin Elmer) was added and the tube placed in a Perkin Elmer 9600 thermal cycler. The tube was then raised to 80°C for 15 seconds followed by cooling to 4°C. A second set of reagents was then added to each tube as below:-

4.0 µl 10x buffer;
9.6 µl sterile deionised water;

0.4 μl (0.5 units) AmpliTaq DNA polymerase (Perkin Elmer);
2 μl DNA lysate.

Reaction tubes were then placed on a Perkin Elmer 9600 thermal cycler preheated to 94°C and PCR carried out according to the regime indicated below:-

94°C for 4 minutes;
20 cycles of 94°C for 30 secs, 62°C for 30 secs and 72°C for 30 secs;
0°C until required.

The number of cycles may vary depending upon the tissue used as the DNA source.

KIT primers

Forward GAATATTGTTGCTATGGTGATCTCC *KIT*1-FOR
Reverse CCGCTTCTGCGTGATCTTCCTG *KIT*1-REV

CRC primers

Forward CTGGATGTCCTGTGTTCCCTGT CRC-FORWARD
Reverse AGGTTTGTCTGCAGCAGAAGCTC CRC-REVERSE

The reverse *KIT* primer and the forward *CRC* primer are labelled with the ABI fluorescent dye FAM at the 5' end.

(iii) **Electrophoresis and Quantitation of DNA Fragments**

1 μl of the PCR was mixed with 2.5 μl of deionised formamide, 0.5 μl of GS350 DNA standards, 0.4 μl blue dextran solution, heated at 90°C for 2 minutes followed by

rapid cooling on ice. 3 μl of this mix were then loaded onto an AB1373 DNA sequencer and DNA fragments separated on a 6% polyacrylamide gel in 1 x TBE buffer for 2 hours at 700 V, 40 mA, 32 W. The fragments corresponding to the products from the *KIT* and *CRC* genes were quantitated using GeneScan software, the peak area for each of the bands being determined.

(iv) **Results**

The data given in Table 1 represents the results obtained from an experiment in which DNA lysates were produced from each of 23 animals, with two PCR tests being carried out on each lysate. The ratio of *KIT* peak area to *CRC* peak area was calculated for each PCR and the average taken of those samples from the same animal.

TABLE 1

Animal	Genotype	KIT/CRC peak area ratio
1	II	3.25
2	Ii	2.45
3	II	2.94
4	ii	1.16
5	ii	1.34
6	ii	1.20
7	Ii	2.18
8	Ii	2.19
9	II	2.88
10	ii	1.30
11	Ii	1.84
12	II	2.84
13	ii	1.50
14	ii	1.30
15	Ii	2.07
16	ii	1.31
17	ii	1.14
18	Ii	2.02
19	Ii	1.87
20	Ii	2.00
21	ii	0.99
22	ii	1.15
23	II	2.80

The upper and lower limits for the ratio values from animals of the different genotypes *II*, *Ii* and *ii* in this experiment are as below:

Genotype	Upper Limit	Lower Limit
I/I	3.25	2.80
I/i	2.45	1.84
i/i	1.50	0.99

These results illustrate differentiation of the genotypes using this test.

EXAMPLE 2

The second test utilises unique sequences of DNA that are present at one end of the duplication (or both ends if the duplicated region is reversed relative to the rest of the gene or if the duplicated region does not occur in direct tandem with the non-duplicated region). Oligonucleotide primers for use in PCR are designed such that at the annealing temperatures used in the PCR process, they will anneal only to the junction regions at the end of the duplicated region. A PCR is then carried out using two pairs of oligonucleotides. One pair consists of the aforementioned primer spanning the junction region and a second primer a suitable distance away which allows amplification to occur only from *I* allele containing duplication. The second pair of primers allow amplification of a sequence present only as a single copy in the haploid genome. The product of this reaction, carried out in the same tube, functions as an internal standard as in the previous test. The ratio of product from the reaction specific to the junction region is measured relative to that from the single copy control sequence.

In this test there is a larger difference between the predicted ratios of the products from the different

genotypes. The relative levels of product and their ratios are illustrated below:-

Genotype	Junction Product	Control Product	Ratio
II	2	2	1:1
Ii	1	2	1:2
ii	0	2	0:2

These larger ratios allow greater differentiation between the ranges of results obtained from the different genotypes, reducing risks of miss-scoring animals.

EXAMPLE 3

(i) DNA Preparation

DNA can be prepared as described in EXAMPLE 1

(ii) PCR

Reactions were set up as follows in thin walled 0.25 ml tubes (Perkin Elmer):

2.0 μl 5 mM *KIT*DEL2-FOR primer;
2.0 μl 5 mM *KIT*DEL2-REV primer;
1.0 μl 2 mM dNTPs (Pharmacia);
1.2 μl 25 mM MgCl2
2.0 μl 10x buffer (without MgCl2)

0.1 μl (0.5 units) AmpliTaq DNA polymerase (Perkin Elmer);
2.0 μl DNA lysate;
9.7 μl sterile deionised water.

Reaction tubes were then placed on a Perkin Elmer 9600 thermal cycler and PCR carried out according to the regime indicated below:-

95°C for 1 minute;
3 cycles of 95°C for 15 secs, 50°C for 20 secs and 72°C for 40 secs;
27 cycles of 94°C for 15 secs, 50°C for 20 secs and 72°C for 50 secs;
72°C for 5 minutes;
4°C until required.

The number of cycles may vary depending upon the tissue used as the DNA source.

KIT primers

Forward GAAAGTGA(C/T)GTCTGGTCCTAT(C/G)GGAT *KIT*DEL2-FOR
Reverse AGCCTTCCTTGATCATCTTGTAG *KIT*DEL2-REV

(iii) Electrophoresis

1 µl of the PCR product was mixed with 3 µl loading buffer (95% deionised formamide, 10mM NaOH, 20mM EDTA, 0.05% bromophenolblue, 0.05% Xylene-cyanol), heated to 95°C for 3 minutes followed by rapid cooling on ice. The sample was then loaded on an 8% native polyacrylamide gel (Protogel, 37.5:1 Acrylamide:bisacrylamide, National Diagnostics, Atlanta) in 1 x TBE buffer (89mM Tris, 89mM boric acid, 2mM EDTA.Na2). The DNA fragments were separated by electrophoresis for 4.5 hours at 6W with a constant temperature of 20°C and 0.6 x TBE as running buffer in a vertical slab unit (SE600 Hoefer Scientific Instruments, San Francisco).

(iv) Visualisation of DNA fragments by silver staining

After electrophoresis the gel was incubated, with gentle agitation, in the fix solution for 20 minutes or until the tracking dyes were no longer visible. The gel was rinsed three times (2 minutes each with agitation) in deionised water. The gel was then incubated in the staining solution for 40 minutes, with gentle agitation, followed by a brief wash (5-10 seconds) in deionised water and direct transfer to the developing solution. The gel was incubated in the developing solution until bands were clearly visible and then the development was terminated by adding an equal volume of fix solution. Finally, the gel was rinsed for 2 minutes in deionised water.

Reagents.

Fix solution:	10% glacial acetic acid in deionised water
Staining solution:	2 g silver nitrate (AgNO3) 3 ml 37% formaldehyde 2 liters deionised water
Developing solution:	60 g sodium carbonate (Na2CO3) dissolved in 2 liters deionised water. Immediately before use add 3 ml 37% formaldehyde and 400 ml sodium thiosulfate (10 mg/ml). The solution should be at a temperature of 10-12°C when used.

(v) Results

This SSCP analysis reveals an informative polymorphism so far only found in animals with the dominant white phenotype (Fig. 1). In lanes 1 to 8 the analysis was carried out on DNA from Swedish Landrace pigs carrying the dominant white colour and in lanes 9 and 10 DNA was from wild pigs of wild type colour. The polymorphic bands are indicated. The polymorphism is characterised by two unique fragments only present in animals carrying a duplicated *KIT* gene of allele type I. The fragments represent heteroduplexes of DNA strands from PCR products of unequal length representing the duplicated and non-duplicated copy of the *KIT* gene. The results of a screening test with this marker using 40 unrelated animals representing five breeds and 190 F2 animals from a Large White/Wild pig intercross are presented in Table 2.

The results show that this particular polymorphism is very closely associated with the presence of the *KIT* duplication. It is not completely associated with the duplication as some white animals did not show the heteroduplex pattern. The polymorphism is therefore an example of a closely linked genetic marker which by itself or in combination with other linked markers can be used to differentiate genotypes as regards the dominant white coat colour.

TABLE 2

BREED	COLOUR	NO. OF ANIMALS	HETERODUPLEX	
			PRESENT	NOT PRESENT
SWEDISH LANDRACE	WHITE	10	10	0
SWEDISH LARGE WHITE	WHITE	8	8	0
SWEDISH HAMPSHIRE	COLOURED	10	0	10
SWEDISH DUROC	COLOURED	10	0	10
WILD PIG	COLOURED	2	0	2
LARGE WHITE/ WILD PIG INTERCROSS	WHITE PATCH COLOURED	131 9 50	106 0 0	25 9 50

EXAMPLE 4

i) DNA extraction

DNA was prepared as in example 1.

ii) PCR

Reactions were set up in 0.25ml thin walled reaction tubes (Perkin Elmer) as follows:

0.5ml	5 μM *KIT*DEL1-FOR primer
0.5ml	5 μM *KIT*DEL1-REV primer
1.0ml	2mM dNTPs (Pharmacia)
1.0ml	15mM $MgCl_2$
1.0ml	10X buffer
4.9ml	Sterile distilled water
0.1ml	AmpliTaq DNA polymerase
1.0ml	DNA lysate

Reaction tubes were then placed in a Perkin Elmer 9600 thermal cycler and PCR carried out according to the regime

94°C for 4 minutes;
21 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec;
72°C for 4 min;
4°C until required.

The number of cycles used may vary depending on the tissue used as the source of the DNA.

Primers

forward	TGTGGGAGCTCTTCTCTTTAGG	*KIT*DEL1-FOR
reverse	CCAGCAGGACAATGGGAACATCT	*KIT*DEL1-REV

The reverse primer was labeled with the ABI fluorescent dye FAM at the 5′ end.

iii) Electrophoresis and quantitation of DNA fragments
1μl of the PCR was mixed with 1.5μl of deionised formamide, 0.25μl of GS350 DNA standards, 0.25μl loading buffer (50mg/ml blue dextran, 25mM EDTA) and heated at 90∘C for two minutes followed by rapid cooling on ice. 1.75μl of this was then loaded onto an ABI 377DNA sequencer and DNA fragments separated on a 4.12% polyacrylamide gel in 1x TBE buffer for two hours at 3000V, 60mA, 200W and 48∘C. The 97bp and 93bp fragments corresponding to the products from the *KIT* gene template lacking the deletion and containing the deletion respectively were quantitated using GeneScan software, the peak area for each of the bands being determined.

Results

The data given in the table below represents the results obtained from an experiment in which DNA lysates were produced from each of 20 animals of known genotype with one PCR test being carried out on each lysate. The ratio of the peak area of the product from the DNA template not containing the four base pair deletion to that containing the deletion was calculated.

TABLE 3

ANIMAL	GENOTYPE	Non del/del peak area ratio
1	*II*	1.347
2	*II*	1.21
3	*II*	1.33
4	*II*	2.267
5	*II*	0.444
6	*II*	0.713
7	*II*	8.387
8	*II*	0.994
9	*II*	1.673
10	*II*	1.056
11	*Ii*	1.751
12	*Ii*	1.73
13	*Ii*	1.83
14	*Ii*	0.631
15	*Ii*	1.975
16	*Ii*	2.147
17	*Ii*	1.901
18	*Ii*	1.749
19	*Ii*	2.103
20	*Ii*	2.026

For this small sample the value of 1.5 which is midway between the predicted ratio values for each genotype (expected ratio=2 for *Ii* and 1 for *II*) might be used as the dividing line for scoring the animals to either genotype. It can be determined from the table that 7/10 *II* and 9/10 *Ii* are identified as the correct genotype.

CLAIMS:

1. A method of determining the coat colour genotype of a pig which comprises:-

(i) obtaining a sample of pig nucleic acid; and

(ii) analysing the nucleic acid obtained in (i) to determine whether duplication of all or part of the *KIT* gene is present.

2. A method as claimed in claim 1 wherein the pig nucleic acid is genomic DNA.

3. A method as claimed in claim 2 wherein step (ii) includes amplification of at least a part of the *KIT* gene by means of one or more cycles of PCR and a pair of suitable primers.

4. A method as claimed in claim 3 wherein the amount of PCR product is quantified.

5. A method as claimed in claim 3 or claim 4 wherein the pair of primers are:

GAATATTGTTGCTATGGTGATCTCC
CCGCTTCTGCGTGATCTTCCTG;

GG(C/T)AATCACATGAATATTGTGAA
TCACCATAGCAACAATATTCTGT;

TC(A/G)TACATAGAAAGAGA(C/T)GTGACTC
CCTTT(A/G)ACCAC(A/G)TAATT(A/C)GAATC; or

GTGATG(A/G)T(G/T)CT(C/G)ACCTACAAATA
GTCTATGTAAACATAATTGTTTCC.

6. A method as claimed in claim 3 or claim 4 wherein the pair of primers hybridise to a unique region located at the boundary of the *KIT* gene and the duplicated region.

7. A method as claimed in any one of claims 3 to 6 wherein the determination includes comparison with an internal standard.

8. A method as claimed in claim 7 wherein the internal standard is generated by amplification of at least a part of another nucleotide sequence by means of one or more cycles of PCR using a pair of suitable primers.

9. A method as claimed in claim 8 wherein the amount of internal standard PCR product is quantified.

10. A method as claimed in claim 8 or claim 9 wherein the internal standard PCR product results from amplification of at least a part of the sequence of the muscle calcium release channel (*CRC*) gene.

11. A method as claimed in claim 10 wherein the following pair of primers is used for the internal standard PCR reaction:

CTGGATGTCCTGTGTTCCCTGT and
AGGTTTGTCTGCAGCAGAAGCTC.

12. A method as claimed in claim 10 wherein the following pair of primers is used for the internal

standard PCR reaction:

GATGAACTTTGAGGTCCCTGAGGAG and TTTCTTCTGAGAATGCCGAAGATCTG.

13. A method as claimed in any one of claims 8 to 12 wherein the internal standard PCR reaction is carried out simultaneously with the amplification of the *KIT* sequence.

14. A method as claimed in any one of claims 8 to 13 wherein the ratio, copies of *KIT* region:copies of internal standard region is determined.

15. A method as claimed in any one of claims 1 to 14 which further comprises the step:

(iii) determining whether any duplication present is due to the presence of *I* or I^P.

16. A method as claimed in claim 14 wherein the presence or absence of a deletion in at least one of the duplicated regions is determined.

17. A method as claimed in claim 16 wherein the ratio, copies of non deleted region:copies of deleted region is determined.

18. A method for determining the coat colour genotype in pigs, which comprises:-

(i) obtaining a sample of pig genomic DNA;

(ii) hybridising the genomic DNA from (i) with one

or more suitable primers;

(iii) carrying out one or more PCR cycles using the hybridised nucleic acid from (ii); and

(iv) determining the amount of PCR reaction product.

19. A method as claimed in claim 18 which further comprises the step:

(v) determining whether any duplication present is due to the presence of *I* or I^P.

20. A method as claimed in claim 19 wherein the presence or absence of a deletion in at least one of the duplicated regions is determined.

21. A method as claimed in any one of claims 18 to 20 modified by any one or more of the features of claims 6 to 14.

22. A method for determining the coat colour genotype of a pig, which method comprises:

(i) obtaining a sample of pig *KIT* protein; and

(ii) analysing the protein obtained in (i) to determine whether duplication of all or part of the *KIT* gene is present.

23. A method as claimed in claim 22 wherein step (ii) is carried out by reacting the protein with one or more antibodies.

24. A method as claimed in claim 23 wherein the method is an ELISA.

25. A kit for determining the coat colour genotype of a pig, comprising one or more reagents capable of indicating the presence of duplication of all or part of the *KIT* sequence.

26. A kit as claimed in claim 25 wherein the kit is adapted to be used with a sample of pig genomic DNA.

27. A kit as claimed in claim 25 or claim 26 comprising reagents for carrying out PCR together with at least one pair of primers.

28. A kit as claimed in claim 27 wherein the pair of primers are:

GAATATTGTTGCTATGGTGATCTCC
CCGCTTCTGCGTGATCTTCCTG;

GG(C/T)AATCACATGAATATTGTGAA
TCACCATAGCAACAATATTCTGT;

TC(A/G)TACATAGAAAGAGA(C/T)GTGACTC
CCTTT(A/G)ACCAC(A/G)TAATT(A/C)GAATC; or

GTGATG(A/G)T(G/T)CT(C/G)ACCTACAAATA
GTCTATGTAAACATAATTGTTTCC.

29. A kit as claimed in claim 27 wherein the pair of primers hybridise to a unique region located at the boundary of the *KIT* gene and the duplicated region.

30. A kit as claimed in any one of claims 26 to 29 further comprising a second pair of primers allowing amplification of at least a part of another nucleotide sequence by means of one or more cycles of PCR.

31. A kit as claimed in claim 30 wherein the second pair of primers hybridise to at least a part of the sequence of the muscle calcium release channel (*CRC*) gene.

32. A kit as claimed in claim 31 wherein the second pair of primers have the sequence:

CTGGATGTCCTGTGTTCCCTGT and
AGGTTTGTCTGCAGCAGAAGCTC.

33. A kit as claimed in claim 30 wherein the second pair of primers hybridise to at least a part of the sequence of the porcine Interferon-β gene.

34. A kit as claimed in claim 33 wherein the second pair of primers have the sequence:

GATGAACTTTGAGGTCCCTGAGGAG and
TTTCTTCTGAGAATGCCGAAGATCTG.

35. A kit as claimed in claim 25 comprising one or more antibodies capable of reacting with the *KIT* protein.

36. A method of determining the coat colour genotype of a pig which comprises:-

(i) obtaining a sample of pig nucleic acid; and

(ii) analysing the nucleic acid obtained in (i) to

identify the presence or absence of a deletion in the *KIT* gene sequence.

37. A method as claimed in claim 36 wherein the pig nucleic acid is genomic DNA.

38. A method as claimed in claim 36 or claim 37 wherein step (ii) includes amplification of at least a part of the *KIT* gene by means of one or more cycles of PCR and a pair of suitable primers.

39. A method as claimed in claim 38 wherein the pair of primers are:

TGTGGGAGCTCTTCTCTTTAGG
CCAGCAGGACAATGGGAACATCT; or

GAAAGTGA(C/T)GTCTGGTCCTAT(C/G)GGAT
AGCCTTCCTTGATCATCTTGTAG.

40. A method as claimed in any one of claims 36 to 39 wherein the method is used to distinguish between *I* and *i*.

41. A kit for determining the coat colour genotype of a pig, comprising one or more reagents capable of indicating the presence or absence of a deletion in the *KIT* sequence.

42. A kit as claimed in claim 41 wherein the kit is adapted to be used with a sample of pig genomic DNA.

43. A kit as claimed in claim 41 or claim 42 comprising reagents for carrying out PCR together with at least one

pair of primers.

44. A kit as claimed in claim 43 wherein the pair of primers are:

TGTGGGAGCTCTTCTCTTTAGG
CCAGCAGGACAATGGGAACATCT; or

GAAAGTGA(C/T)GTCTGGTCCTAT(C/G)GGAT
AGCCTTCCTTGATCATCTTGTAG.


1. 2. 3. 4. 5. 6. 7. 8. 9. 10

INTERNATIONAL SEARCH REPORT

Intern   nal Application No
PCT/GB 96/01794

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   C12Q1/68   C07H21/04   C12P19/34   G01N33/53   G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GENOMICS, vol. 14, December 1992, pages 965-69, XP000609787 JOHANSSON J ET AL: "The gene for dominant white color in the pig is closely linked to ALB and PDGFRA on chromosome 8" cited in the application see page 967, paragraph 4; figure 968 ---	1,2
A	ONCOGENE, vol. 9, September 1994, pages 2655-61, XP000608008 SEPULVEDA P ET AL: "Instability at the W/c-kit locus in mice: analysis of melanocyte cell lines derived from reversion spots " see the whole document ---	1-6, 25-29, 41-44

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[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search	Date of mailing of the international search report
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Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Osborne, H

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CIBA FOUNDATION SYMPOSIUM, vol. 148, 1990, pages 158-66, XP000607999 BERNSTEIN A ET AL: "The mouse w/c-kit locus" see the whole document ---	1,2,36, 37,41,42
A	PROCEEDING OF THE NATIONAL ACADEMY OF SCIENCE USA, vol. 88, December 1991, pages 10885-89, XP002018767 FLEISCHMANN R ET AL: "Deletion of the c-kit protooncogene in human developmental defect piebald" see the whole document ---	1,2,36, 37
A	CELL, vol. 55, October 1988, pages 185-92, XP002018768 GEISSLER E ET AL: "The dominant-white spotting (W) locus of the mouse encodes the c-kit proto-oncogene " cited in the application see the whole document -----	1,2,36, 37,41,42

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